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(54) Title: POTENTIATION OF CYTOTOXIC CONJUGATES

(57) Abstract

Cancer therapy and, more particularly, the potentiation or enhancement of the cytotoxic properties of immunotoxins by the co-administration of a second, unconjugated monoclonal antibody.

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POTENTIATION OF CYTOTOXIC CONJUGATES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to cancer therapy and, more particularly, to the potentiation or enhancement of the cytotoxic properties of immunotoxins by the co-administration of a second, unconjugated monoclonal antibody.

Colorectal cancer is the second most common cause of death from malignancy in the Western world. The American Cancer Society estimates that there were 138,000 new cases of colorectal cancer and 59,900 patients died from the disease in 1985. The outlook for patients with colorectal disease has remained essentially unaltered over the last 30 years, with the five-year survival being on the order of 30%. A major factor contributing to this is the lack of effective treatment for the disease once it has spread beyond the bowel wall, since surgery during the early stages of the disease offers the only prospect of cure.

Unfortunately, the majority of patients have disseminated disease at the time of initial surgery, primarily with overt or occult hepatic and lymph node metastases.

Despite numerous trials of chemotherapy, the only single agent shown to have any significant effect is 5-fluorouracil; its response rate is inadequate and treatment with it rarely influences the ultimate outcome. Combination chemotherapy and intrahepatic arterial infusion are being investigated but currently do not represent effective treatments.

Cancer of the ovary accounts for roughly 5% of all cancers in women and is the sixth leading cancer in women. Although surgery is curative if this lesion

is detected early enough, the mortality associated with this disease has not improved appreciably in the last 25 years.

Ultrasound, laparoscopy or peritoneoscopy, and CAT scan are of limited value in the diagnosis of ovarian carcinoma. Serum markers such as carcinoembryonic antigen and placental alkaline phosphatase as well as some newly defined antigens are found in the blood of some patients with adenocarcinoma, although there is no universal marker. Surgery with biopsy is the only definitive way of diagnosing ovarian carcinoma.

Surgery is currently the only cure for ovarian carcinoma, and is only curative if the tumor has not spread. Radioisotope implants, x-ray irradiation, and chemotherapy are of limited use in the management of ovarian carcinoma.

The most important prognostic indication is the extent of spread of the tumor at the time of diagnosis and surgery. Stage I ovarian carcinoma (growth limited to the ovaries) has an overall 5-year survival rate of approximately 80%, Stage II (growth involving ovaries with pelvic extension) has a 5-year survival rate of 40%, Stage III (growth involving ovaries with extension to small bowel or omentum) has a 5-year survival rate of 10%, and Stage IV (distant metastases) has a 5-year survival rate of less than 5%. The relative 5-year survival rate for ovarian carcinoma is 37% (1973-80), relatively the same as for 1960-63 (32%).

Imaging studies of ovarian carcinoma with radiolabelled MoAbs have been performed to a limited degree in human and animal systems. Tumors as small as 1 mm in diameter in mice bearing xenografts of human ovarian cancer have been imaged. The same study also detected tumors in 8 of 10 patients with ovarian cancer.

Osteogenic sarcoma (OS) is the most common primary bone tumor. Although surgery can be curative if this lesion is detected early enough, the usual course of this disease in 80%-85% of patients is multiple

pulmonary metastases and death within two years of diagnosis. These metastases are often present but usually not large enough to be seen at diagnosis of the primary tumor.

Amputation is the treatment of choice for OS. Limb salvage procedures have been performed, such as en-block resection and prosthetic replacement. Overall survival for limb salvage is either as poor or worse than with amputation.

Radiation treatment has not been shown to prevent OS metastases.

Most recent trials note survival rates of over 50% at 5 years for patients treated by widely diverse adjunctive methods. The question is whether all of these widely diverse forms of treatment are effective or if there is a change in the natural history of the disease.

Imaging studies of OS with radiolabelled MoAbs have been performed to a limited degree in human and animal systems. Human OS xenografts have been imaged in nude mice using an anti-OS MoAb labelled with ^{131}I.

Because of the extent of these three forms of cancer, there is a need for new compositions and methods to treat primary, recurrent and metastatic disease.

Description of the Relevant Literature

Ramakrishnan and Houston, Science (1984) 223:58-61, describe the potentiation of immunotoxins directed against human acute lymphoblastic leukemia by chloroquine.

Akiyama et al., Cancer Res. (1985) 45:1005-07, report the potentiation of the cytotoxic activity against human tumor cells of toxic conjugates of Pseudomonas exotoxin with anti-transferring receptor antibody or epidermal growth factor up to 10 to 20 fold by the calcium antagonists verapamil, D-600, and diltiazem and by the lysosomotropic agent ß-glycylphenylnaphthylamide.

Uckun et al., Blut (1985) 50:19-23 describe the potentiation by mafosfamid (ASTA Z 7557) of the ex vivo efficacy of a T-cell directed immunotoxin containing pokeweed antiviral protein (PAP).

Erlich et al., Mixing two monoclonal antibodies yields enhanced affinity for antigen, J. Immun. 128:2709-2713 (1982) describes an increase in sensitivity in antigen-binding assays using a mixture of monoclonal antibodies.

Moyle et al., Quantitative explanation for increased affinity shown by mixtures of monoclonal antibodies: importance of a circular complex, Molec. Immun. 20: 439-452 (1983) describes a mathematical model to predict the quantity of intermediates formed when pairs of IgG1 mouse monoclonals are mixed with human chorionic gonadotropin.

Wellerson R. and Kaplan, P., Enhanced binding activity observed between anti-carcinoembryonic monoclonal antibodies, Hybridoma 5:199-213 (1986) describe the enhancement of binding when the antigen is exposed to a particular mixture of monoclonal antibodies.

SUMMARY OF THE INVENTION

The described invention relates to potentiating the cytotoxicity of antibodies conjugated to a toxin, ie., an immunotoxin. The enhancement of cytotoxicity is demonstrated by a cytotoxic cocktail containing an immunotoxin to which is added a second immunoglobulin that binds to a second epitope on the same antigen to which the immunotoxin binds.

More specifically there is described herein a method for enhancing the cytotoxicity of an immunotoxin consisting of a first immunoglobulin conjugated to a toxin, said first immunoglobulin directed against a cell surface antigen, preferably a tumor associated antigen, wherein the method comprises exposing a target cell expressing the antigen with the immunotoxin and a

second immunoglobulin in a dose sufficient to potentiate said immunotoxin, said second immunoglobulin able to bind to a different epitope on the antigen than that bound by said first immunoglobulin. It is preferred that the first and second immunoglobulins are able to bind to an epitope on a carcinogenicembryonic antigen. Most preferably the epitope bound by the second immunoglobulin is within the normal cross-reacting antigen subsection of the carcinogenicembryonic antigen. Immunoglobulins binding to T-cells both normal and leukemic are useful in the disclosed method. Particularly useful are immunoglobulins to the CD-5 antigen. Specific and preferred monoclonal antibodies for use in this invention and their hybridomas are also described herein.

In addition to the above methods, there is described herein a cytotoxic therapeutic cocktail comprising: a conjugate including a toxin bound to a first immunoglobulin, said first immunoglobulin able to bind to an epitope on a cell surface antigen, preferably a tumor associated antigen; and a second immunoglobulin in a dose sufficient to potentiate said immunotoxin, said second immunoglobulin able to bind to a different epitope on the antigen than that bound by said first immunoglobulin. It is preferred that the cytotoxic therapeutic cocktail embrace first and second immunoglobulins that are able to bind to an epitope on the carcinogenicembryonic antigen. It is most preferred that the second immunoglobulin bind to an epitope that is located within the normal cross-reacting antigen subsection of the carcinogenicembryonic antigen. Cocktails containing immunoglobulins binding to T-cells both normal and leukemic are also useful in this invention. Particularly useful are immunoglobulins to the CD-5 antigen. It should be noted that it is not necessary to physically combine the immunotoxin and second immunoglobulin. Separate administration would also constitute a cocktail as defined herein.

In addition to the above method and cocktails, there is described herein a method of killing cancer cells in humans by enhancing the cytotoxicity of an immunotoxin, which consists of a first immunoglobulin conjugated to a toxin said immunoglobulin directed against a tumor associated antigen wherein the method comprises exposing cancer cells expressing the antigen to the immunotoxin and to a second immunoglobulin in a dose sufficient to potentiate said toxin, said second immunoglobulin able to bind to a different epitope on the antigen than that bound by said first immunoglobulin. It is also preferred that the first and second immunoglobulins are able to bind to an epitope carcinogenicembryonic antigen. It is most preferred that the epitope bound by the second immunoglobulin is within the normal cross-reacting antigen subsection of the carcinocembryonic antigen. This method is useful against cancer cells selected from the group consisting of colorectal carcinoma cells, gastric cancer cells, pancreatic cancer cells, lung cancer cells, and breast cancer cells. Immunoglobulins binding to T-cells both normal and leukemic are useful in the disclosed method. Particularly useful are immunoglobulins to the CD-5 antigen. Preferred dosages are approximately 0.01 mg to 20.0 mg per kg of host body weight per day and most preferably the doses are approximately 0.05 mg to 5.0 mg per kg of host body weight per day. The preferred ratio of said second immunoglobulin to said immunotoxin is 10,000 - 0.001 to 1 by weight and most preferably the ratio of second immunoglobulin to said immunotoxin is 100 - 0.01 to 1 by weight.

By a second epitope it is meant that the two antibodies bind to physically different locations on their target antigen. It is preferred that the binding not be competitive but a slight amount of competition due to close proximity is acceptable. Potentiation can be quantified and the effect of competition may be

offset by appropriate changes in the ratios of immunotoxin to immunoglobulin. Monoclonal antibodies are preferred for use in this invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphic illustration of the influence of free RTA and XMMCO-228-RTA on the growth of colorectal carcinoma LS174T cell xenografts.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention employs cytotoxic conjugates for the treatment of various forms of cancer, including colorectal carcinoma, ovarian carcinoma and osteogenic sarcoma. These novel conjugates are comprised of monoclonal antibodies or binding fragments thereof, collectively termed immunoglobulins, bound to a cytotoxin. These compositions are administered to a cancer cell host in order to destroy cancer cells while doing minimal damage to normal tissue.

The immunoglobulins of the present invention are employed as targeting agents for directing cytotoxic agents to specific cancer cells within a cancer cell host. According to the present invention, immunoglobulins which define an epitope on a 72 kilodalton (kD) glycoprotein antigen and immunoglobulins which define an epitope on carcinoembryonic antigen (CEA) are employed as targeting agents. One or both of these antigens are present on a variety of cancers including, but not limited to, colorectal carcinoma, ovarian carcinoma and osteogenic sarcoma.

A variety of cytotoxic agents are suitable for use in immunotoxins. The cytotoxic agents contemplated by this invention can include radionuclides, such as Iodine-131, Yttrium-90, Rhenium-188, and Bismuth-212; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, and cisplatinum; and cytotoxic proteins such as ribosomal inactivating

proteins including, pokeweed antiviral protein, abrin and ricin (or their A-chains), diphtheria toxin pseudomonas exotoxin A or recombinant derivitives, etc. See generally, "Chimeric Toxins", Olsnee and Phil. Pharmac. Ther., 25:355-381 (1982), and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press (1985), both of which are incorporated herein by reference.

Toxic lectins are of particular interest in this invention. The cytotoxic action of toxic lectins, and especially that of ricin and abrin, has been well studied. It is known that toxic lectins consist of two polypeptide chains, A and B, linked by means of disulfide bridge(s). Cytotoxicity is associated with the A chain and its inhibition of protein synthesis in nucleated cells. The B chain is essentially a delivery vehicle for the A chain. The B chain recognizes polysaccharide units at the surface of cells and creates a high affinity interaction with such units. Once the B chain binds with polysaccharide units at the cell surface, the A chain is incorporated into the cell, blocking ribosomal protein synthesis and ultimately leading to cell death. The use of ricin A chain is preferred in this invention and its use is as described in U.S. Patent No. 4,590,071, the disclosure of which are hereby incorporated by reference.

Although not used in the examples provided, a preferred form of ricin toxin A chain for use in this invention is one wherein substantially pure RTA-30 is used. The term "RTA-30" refers to a species of ricin toxin A chain having a molecular weight of approximately 30 kD, such as described in detail by Fulton et al. J. Biol. Chem., 281:5314-5319 (1986) and Vidal et al. Int. J. Cancer, 36:705-711 (1985). For the purposes of this invention, RTA preparations containing concentrations of about 75% or more of RTA-30 are considered substantially pure. Preparation of substantially pure RTA-30

for use in conjunction with a MoAb is described in U.S. Patent Application Serial Number 074,824 which is incorporated herein by reference.

The immunoglobulin and toxin are generally bound by a covalent bond, more particularly a disulfide bond, but may be joined by any chemical bond which allows the toxin to travel to the target cell with the immunoglobulin. Other methods for achieving such a bond are well-known to those skilled in the art. The only criteria is that the bond must be achieved in a manner which does not significantly decrease the binding affinity of the immunoglobulin for its epitope.

The present cytotoxic conjugates may be administered to a cancer cell host either singly or in a cocktail containing two or more conjugate formulations, other chemotherapeutic agents, compositions, or the like. Cocktails are particularly important in the treatment of heterogeneous tumor cell populations wherein targeting of multiple antigens is critical.

The cytotoxic conjugates of the present invention may be administered to a cancer cell host by any convenient method. Pharmaceutical compositions employing the subject conjugates may be administered parenterally, i.e., intravenously, intraparitoneally, or the like. Thus, this invention provides compositions for parenteral administration which comprise a solution of pyrogen free, cytotoxic conjugates, or a cocktail thereof, dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, or the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well-known filtration sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents,

toxicity adjusting agents, or the like, for example, sodium acetate, sodium chloride, potassium chloride, potassium chloride, calcium chloride, sodium lactate, etc.

The compositions of the present invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss and that use levels may have to be adjusted to compensate.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulation should provide a quantity of conjugate(s) of this invention sufficient to effectively treat the cancer cell host. The dose of conjugate or conjugate cocktail will vary widely, generally from about 0.01 mg/kg/day to 20.0 mg/kg/day, usually about 0.05 mg/kg/day to 10.0 mg/kg/day, and, more particularly, 0.05 mg/kg/day to 5.0 mg/kg/day.

The dose of unconjugated immunoglobulin will vary widely depending upon the immunotoxin employed, choice of unconjugated immunoglobulin, nature and extent of the tumor, and the like. The dose will generally be in the range of 0.001 mg/kg/day to 100.00 mg/kg/day, usually about 0.01 mg/kg/day to 10.0 mg/kg/day and, more particularly, about 0.1 to 1 mg/kg/day. The ratio of unconjugated immunoglobulin to conjugate will also vary widely, generally from about 10,000-0.001 to 1, usually about 1000-0.01 to 1 and, more particularly, about 100-0.1 to 1.

Kits may also be supplied for use with the subject conjugates or cocktails. Thus, the subject

compositions may be provided, usually in lyophilized form, either alone or in conjunction with additional chemotherapeutic agents for cancer therapy. These kits may include buffers, such as phosphate buffered saline, other inert ingredients, or the like. The kits may also include specific instructions including suggested protocols for the administration of the subject compositions to a cancer cell host.

While the actual mechanism of endocytosis is poorly understood, it is known that most, if not all, immunotoxins must be endocytosed, following binding to target cell surface antigens, in order to be efficacious. It is believed that endocytosis is triggered by the binding of receptors on cell surface antigens.

According to the present invention, the efficacy of the subject immunotoxin compositions may be potentiated by the co-administration, via a similar route, of an unconjugated immunoglobulin in conjunction with the subject immunotoxins. Potentiation means that the efficacy of the immunotoxin, in terms of target cell kill, is increased for a given dose of immunotoxin.

Although the following theory should not be deemed a limitation of this invention, it is believed that the enhanced cytotoxicity is due to an increased affinity for the antigen caused by the binding of the second immunoglobulin. This rationale will have wide ranging effects upon the use of immunoconjugates where the target cell antigen is defined and antibodies binding to related epitopes are available.

The following examples are offered by way of illustration and not limitation.

EXPERIMENTAL

EXAMPLE I - XMMCO-228-RTA CONJUGATES

A. Production of Hybridomas

Balb/c mice (Bantin & Kingman, U.K.) were immunized with carcinoembryonic antigen (CEA) derived from a perchlorate extract of a colon carcinoma liver metastasis. The immunization schedule consisted of 10 µg CEA in complete Freund's adjuvant (CFA) given intraperitoneally on Days 0 and 7, and 20 µg of CEA (Also in CFA) given intraperitoneally on Days 25, 56 and 63.

Three days after the last antigen boost, spleen cells from an immunized mouse were aseptically removed. Following procedures as outlined elsewhere (Galfre et al (1977) Nature 266:550, which is incorporated by reference), 10^6 spleen cells were fused with 10^6 cells of P3-NSI-Ag4-1 (A.T.C.C. Accession No. TIB-18), a hypoxanthine-methotrexate-thymidine (HMT) sensitive murine myeloma cell line. Using polyethylene glycol (PEG), hybrid cells were placed into 96-well culture plates, (Costar, Cambridge, MA #3596) on medium containing a feeder layer of rat peritoneal exudate cells (2.5×10^3 cells/well). Cells were cultured in Dulbecco's Modified Eagle's Medium (Flow Labs, Irvine, U.K.) containing 15% Fetal Calf Serum (Myoclone, Gibco, Paisley, U.K.) and hypoxanthine (10^{-4} M), thymidine (1.6×10^{-5} M) (both from Sigma, Dorset, U.K.) and methotrexate (10^{-5} M) (Lederle, Hampshire, U.K.).

Within two weeks post fusion, cultures of hybridoma cells were tested for antibody binding to CEA by enzyme immunoassay (EIA). Cultures that were positive were cloned using limiting dilution, plating 1-3 cells/well into 96 well culture plates. Wells containing only one colony were identified by microscopic examination, then tested for reactivity with CEA and normal colon antigen (NCA) by EIA and radioimmunoassay

(RIA), and for reactivity on extranuclear membranes from primary colorectal tumors and normal colon by EIA. The clone designated XMMCO-228 was found to stably secrete immunoglobulin which was determined to be of the IgG2a subclass by solid phase RIA using standard methods. Hybridoma XMMCO-228 is presently on deposit with the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Dr., Rockville, MD 20852, USA. The deposit was made on August 14, 1986, and given A.T.C.C. Accession No. HB 9174.

Balb/c mice (Bantin & Kingman, U.K.), 6-10 weeks old, were used to culture the hybridoma peritoneally. Approximately 10^7 hybridoma cells were injected into mice that had been pretreated 3 weeks earlier with 0.5 mls of pristane (Aldridge, Gillingham, Dorset, U.K.) injected intraperitoneally (i.p.). The resultant ascites fluid, collected 3 weeks after injection of the hybridomas, contained on average 5 mg/ml of the antibody as determined by measuring immunoglobulin level according to the method of Price and Baldwin ICRS Med. Sci. (1984) 12:1000-01, which is incorporated by reference.

The antibody in ascites fluid was purified by affinity chromatography using a Sepharose - Protein A column using methods well known by those skilled in the art.

The hybridoma was grown in vitro in Dulbecco's Minimal Essential Medium (Flow Labs, Irvine, U.K.) with 10% Fetal Calf Serum (Myoclone, Gibco, Paisley, U.K.) and hypoxanthine (10^{-4} M), thymidine (1.6×10^{-5} M) (both from Sigma, Dorset, U.K.) and methotrexate (10^{-5} M) (Lederle, Hampshire, U.K.) in plastic 300 ml bottles. Cell concentration was 10^5 cells/ml medium over a culturing period of 4-5 days, with a MoAb concentration of 4 µg/ml medium, and a doubling time of 12 hours.

B. In Vitro Antibody Binding to Cell Lines Measured by Flow Cytometry

The binding of XMMCO-228 to MKN45 cells was determined by flow cytometry employing methods well known to those skilled in the art and described above. The gastric carcinoma cell line MKN45 expresses both the 72kD antigen with which the XMMCO-791 MoAb reacts and the CEA antigen with which XMMCO-228 reacts. B14 is a MoAb directed against breast cancer, which reacts with the normal cross-reacting antigen (NCA) subsection of the CEA antigen. It is described in Example II. Normal mouse serum was used as a control. The results are summarized in Table I.

TABLE I

Binding to MKN45 Cells by Flow Cytometry

MoAb	Mean Fluorescence Intensity
XMMCO-228	987
B14	790
Control	15

C. In Vivo Antibody Binding to Normal Tissues Measured by Immunoperoxidase

XMMCO-228 was evaluated for crossreactivity with normal tissues using the indirect immunoperoxidase conjugate technique. As a control, tissues were simultaneously tested with a purified murine MoAb in the same concentration. The control immunoglobulins used for these purposes were an IgG2 mouse myeloma protein (UPC 10, Litton Bionetics, Kensington, MD) or a MoAb with reactivity to sheep red blood cells (SRBC).

Fresh tissue slices (2-3mm thickness and up to 2.0 cm in width) was generously coated with Optimal

Cutting Temperature Compound (OCT). (Ames Co., Elkhar, Indiana) and wrapped in aluminum foil. The specimen was "snap" frozen in liquid nitrogen-cooled isopentane (2-methylbutane, VWR, Norwald, CA) at controlled temperature (-120 to -150°C) for 10-15 seconds. The tissue was kept frozen for long-term storage at -70°C, or for short-term storage at -20°C, in a tightly sealed container to prevent evaporation and drying of tissues.

Frozen tissues were allowed to equilibrate slowly overnight by placing in a -35°C cryostat prior to cutting the frozen sections. Cut sections were dried overnight at room temperature prior to staining.

A circle was etched on each slide around the tissue using a diamond pencil. Slides were then fixed in acetone for 1 minute, followed by a 10 minute PBS wash. Excess fluid was removed and the primary antibody applied for one hour while making sure that the tissue was covered and not allowed to dry out, followed by a 10 minute PBS wash.

Excess fluid was removed and peroxidase-conjugated goat anti-mouse IgG (Tago/product code 6450) (diluted 1:10 in PBS) applied for 30 minutes, followed by a 10 minute PBS wash.

The slides were developed in aminoethylcarbazole (AEC), (0.5 ml AEC stock 120 mg AEC in 15 ml dimethylformamide), 9.5 ml acetate buffer (79 ml of 0.1 M sodium acetate, 21 ml of 0.1 N acetic acid), and 0.05 ml 3% H_2O_2) for 5 minutes, followed by a 5 minute H_2O wash. The slides were then counterstained with fresh Mayer's Hemtoxylin for 5 minutes, followed by a 5 minute wash in running tap water and coverslipped with glycerine mounting media. Table II is a summary of the results:

TABLE II

Antibody Binding to Normal Tissues

Measured by Immunoperoxidase

COLORECTAL CANCER:	5/5 intense staining of Ca 90%+ of population staining +)
NORMAL COLON:	5/5 intense staining of glandular epith., especially intense apical staining
AUTOPSY TISSUE:	Negative tissue-
	Kidney Pancreas Muscle Liver Skin Heart Testis Spleen Spinal Cord Stomach Aorta Cerebellum Lymph Node
	Questionable (+/-)
	Lung
	Positive-
	Esophagus(+ squamous epith)

NOTE: glucose oxidase method showed that Ab. 228 does not stain granulocytes. Almost every other antibody that we have studied that is similar to CEA has stained granulocytes.

It is significant that XMMCO-228 MoAbs do not bind to granulocytes. Normal colon antigen (NCA) appears on such diverse tissues as normal colon, granulocytes, and progenitor bone marrow cells. Because the CEA antigen has a NCA subsection, many anti-CEA MoAbs also bind the NCA on various normal tissues, which XMMCO-228 MoAbs do not.

D. XMMCO-228 Ricin Toxin A Chain (RTA) Conjugates

The conjugation technique, including purification of the A chain of ricin, is disclosed in U.S. Patent No. 4,590,071, the disclosures of which are hereby incorporated by reference.

E. XMMCO-228-RTA In Vitro Cytotoxicity

In vitro cytotoxicity of XMMCO-228-RTA conjugates was determined by the ^{75}Se-Selenomethionine Assay. Single cell suspensions of gastric carcinoma derived MKN45 cells were inoculated with immunotoxin or RTA for 15 minutes, washed and added to flat bottomed microtiter plates at a concentration of 10^5 cells/0.2 ml of immunotoxin or RTA in RPMI medium containing 10% fetal calf serum. Various amounts of immunotoxin were added to each well. Twenty-four hours later, ^{75}Se-Selenomethionine was added to each well. Cultures were further incubated for 16 hours, washed, dried on the bottom of the wells, and the wells were counted in a gamma counter. The results are summarized in Table III, expressed in terms of 50% inhibition concentration.

TABLE III

In Vitro Cytotoxicity Against MKN45 Cells

Agent	IC_{50} (ng/ml)
RTA	2238
XMMCO-228-RTA	600

Agent	IC_{50} (molarity)*
RTA	1×10^{-8} M
XMMCO-228-RTA	1×10^{-10} M

*Adjusted for equivalent RTA molarity

F. XMMCO-228-RTA Therapy of Human Tumor Xenografts

For each data point, 7-10 nude mice were implanted with LS174T cells, a human colon adenocarcinoma derived cell line (A.T.C.C. Accession No. CL 188). Three days post-implant, the first dose of the treatment compound was administered, followed by 10-20 doses until the schedule dose was completed. The results are summarized in Table IV, expressed as a ration of the tumor weight in treated mice divided by the tumor weight in control (untreated) mice. The results, in terms of mean tumor diameter, are summarized graphically in Figure I.

TABLE IV

Therapy of Human Tumor Xenografts
With XMMCO-228-RTA

Expt.	Xenograft Tumor	Treatment Compound	Schedule Dose mg/kg	Response: Tumor Weight Treated / Tumor Weight Control
1	LS 174T	RTA	20	0.70
2	LS 174T	XMMCO-228	80	1.18
3	LS 174T	XMMCO-228-RTA	56	0.36

These results demonstrate the efficacy of XMMCO-228-RTA in the therapy of human tumor xenografts.

EXAMPLE II - XMMCO-791-RTA AND XMMOC-228-RTA COCKTAILS

A. XMMCO-791-RTA + XMMCO-228-RTA Conjugate Cocktails

A cocktail of XMMCO-791-RTA (Scand. J. Immuno. 18:411-420 (1983) and XMMCO-228-RTA consists of a mixture of XMMCO-791-RTA and XMMCO-228-RTA, in any ratio, and administered to a cancer cell host in a dose range of about 0.01 mg/kg/day to 20.0 mg/kg/day. The cocktail is administered parenterally, generally by intravenous infusion or intrapercitoneal injection in a suitable vehicle, such as phosphate buffered saline or the like.

B. In Vitro Cytotoxicity of Conjugate Cocktails

In vitro cytotoxicity of XMMCO-228-RTA conjugates was determined by the ^{75}Se-Selenomethionine assay. Single cell suspensions of gastric carcinoma derived MKN45 cells were inoculated with immunotoxin or RTA for 15 minutes, washed and added to flat bottomed microtiter plates at a concentration of 10^5 cells/0.2 ml of immunotoxin or RTA in RPMI medium containing 10% fetal calf serum. Various amounts of immunotoxin were added to each well. Twenty-four hours later,

^{75}Se-Selenomethionine was added to each well. Cultures were further incubated for 16 hours, washed, dried on the bottom of the wells, and the wells were counted in a gamma counter. The results are summarized in Table IV above, expressed in terms of 50% inhibition concentration.

These data indicate that when XMMCO-791-RTA and XMMCO-228-RTA are administered in a "cocktail" form, the cytotoxicity is at least additive, and possibly synergistic. They also show the efficacy of immunotoxin (or antibody) directed RTA compared to RTA alone.

C. XMMCO-791-RTA + XMMCO-228-RTA Conjugate Cocktail Therapy of Human Tumor Xenografts

For each data point, 7-10 nude mice were implanted with MKN 45 cells, derived cell line. Three days post-implant, the first dose of the treatment compound was administered, followed by 10-20 doses until the scheduled dose was completed. The results are summarized in Table V, expressed as a ratio of the tumor weight in treated mice divided by the tumor weight in control (untreated) mice.

TABLE V

Therapy of Human Tumor Xenografts

With XMMCO-228-RTA

Expt.	Xenograft Tumor	Treatment Compound	Schedule Dose mg/kg	Response: Tumor Weight Treated / Tumor Weight Control
1	MKN 45	XMMCO-228-RTA	25	0.74
		XMMCO-228	25	1.1
2	MKN 45	XMMCO-228-RTA + XMMCO-791-RTA	*	0.56

*25 mg/kg each of XMMCO-228-RTA and XMMCO-791-RTA administered as a mixture.

These data indicate that when XMMCO-791-RTA and XMMCO-228-RTA are administered in a "cocktail" form, the cytotoxicity is at least additive, and possibly synergistic. They also show the efficacy of a cocktail consisting of XMMCO-791-RTA and XMMCO-228-RTA in treating cancer cells in a host.

EXAMPLE III - POTENTIATION OF XMMCO-228-RTA BY XMMBR-B14 MOABS

A. Production of XMMBR-B14 Hybridomas

Balb/c mice were immunized with 521AM whole cells derived from ascites breast metastasis. The immunization schedule consisted of 6 intraperitoneal injections of 10^6 cells at weekly intervals. A final intravenous boost of $3x10^5$ cells was given 3 days prior to fusion.

Three days after the last antigen boost, spleen cells from an immunized mouse were aseptically removed. A single cell suspension was obtained using an 80 mesh wire screen grid #1985-00080 (Bellco). Cells were washed with Iscoves Complete Medium (Grand Island, N.Y., N.Y.)

and counted. Sp2/0-Ag14 cells (A.T.C.C. Accession No. CRL 1581), a hypoxanthine-aminopterin-thymidine (HAT) sensitive murine myeloma cell line, were washed 3 times and counted. Using polyethylene glycol (PEG 4000 Merck) 30% v/v, 10% DMSO and 60% Iscoves, the two cell types were fused at a ratio of 2 spleen cells per myeloma cell. The fusion products were plated into 96-well culture plates at a concentration of 10^5 myeloma cells per well. Cells were cultured in Iscoves with 20% fetal bovine serum and ß mercaptoethanol with HAT medium (hypoxanthine 136 mg/100 ml, aminopterin .018 mg/100 ml, thymidine 136 mg/100 ml).

Within two weeks post fusion, cultures of hybridoma cells were tested for antibody binding to 521 AM tumor cell membranes by dotting onto nitrocellulosee membranes at 200 ng using a Vector Labs ABC kit to test for reactivity. Wells that gave a blue dot were then screened against fibroblast membrane extracts from the same patient. Cultures that were positive were cloned using limiting dilution, plating 1-3 cells/well into 96 well culture plates. Wells containing only one colony were identified by microscopic examination, then tested for reactivity. The clone designated XMMBR-B14 was found to stably secrete immunoglobulin which was determined to be of the IgG1 subclass. Hybridoma XMMBR-B14 is presently on deposit with the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Dr., Rockville, MD 20852, USA. The deposit was made on January 14, 1987, and given A.T.C.C. Accession No. HB 9308.

Balb/c mice (Bantin & Kingman, U.K.), 6-10 weeks old, were used to culture the hybridoma peritoneally. Approximately 10^7 hybridoma cells were injected into mice that had been pretreated 3 weeks earlier with 0.5 mls of pristane (Aldridge, Gillingham, Dorset, U.K.) injected intraperitoneally (i.p.). The resultant ascites fluid, collected 3 weeks after injection of the hybridomas, contained on average 5 mg/ml of the antibody

as determined by measuring immunoglobulin level according to the method of Price and Baldwin ICRS Med. Sci. (1984) 12:1000-01, which is incorporated by reference.

The antibody in ascites fluid was purified by affinity chromatography using a Sepharose - Protein A column using methods well known by those skilled in the art.

The hybridoma was grown and cloned in vitro in Iscoves Medium with 20% fetal bovine serum and β mercaptoethanol in plastic 300 ml bottles. Cell concentration was 10^5 cells/ml medium over a culturing period of 4-5 days, with a MoAb concentration of 4 µg/ml medium, and a doubling time of 12 hours.

B. In Vitro XMMBR-B14 Binding to Cells Measured by Flow Cytometry

The binding of XMMBR-B14 to cell lines and primary carcinoma-derived cells was determined by flow cytometry employing methods well known by those skilled in the art and described above. The tests (Table VI) showed that XMMBR-B14 detects an antigen expressed on breast and colon carcinoma cell lines. Further studies using purified protein preparations have established that the epitope defined by XMMBR-B14 is found on an antigen of the classification termed CEA/NCA (carcinoembryonic antigen/normal cross-reacting antigen). This is the portion of the CEA molecule that also cross-reacts with exposed normal cross-reacting antigen. It also reacts with cells derived from two primary colon carcinomas. Normal mouse immunoglobulin (NMIg) or normal mouse serum (NMS) were used as controls.

TABLE VI

Binding to Various Cells by Flow Cytometry

Target Cell.	Reagent	Fluorescence Units/cell	Reaction
1. Cell Lines			
Colon Carcinoma C170 (Low CEA)	NM Ig	19.3	-
	B14B8	60.4	±
Colon Carcinoma LS174T (Line 1)	NM Ig	22.2	-
	B14B8	2641.7	4+
Colon Carcinoma LS174T (Line 2)	NM Ig	18.6	-
	B14B8	1665.6	3+
Breast Carcinoma BT474-3	NM Ig	17.3	-
	B14B8	316.4	1-2+
2. Primary Colon Carcinoma - Derived Cells			
C212	NMS	31.4	-
	B14B8	607.5	3+
C213	NMS	33.8	-
	B14B8	836.8	4+

C. XMMBR-B14 Reactivity With Colon Carcinoma and Normal Colonic Mucosa Measured by Enzyme Immunoassay (EIA)

XMMBR-B14 was tested for reactivity with primary colon carcinoma membrane and normal colonic mucosa using standard EIA methods well known by those skilled in the art. The test, summarized in Table VII, shows that XMMBR-B14 reacts with colon carcinoma membrane.

TABLE VII

Binding to Colon Membrane by EIA

Reagent	ELISA Units (OD)			
	Normal Colon membrane (NP_1)	Colon Carcinoma Membrane (TP_1)	(T186)	CEA Prep. (B4058)
XMMBR-B14	0.130	0.581	0.758	0.665

TP_1 - membrane preparation from pooled primary colon carcinoma.

NP_1 - membrane preparation from pooled normal colonic mucosa (from colon cancer patient).

T186 - membrane preparation - primary colon carcinoma T186.

CEA(B4058) - CEA preparation - semi-purified from liver metastasis of colon carcinoma

D. XMMBR-B14 Binding to CEA and NCA

The Reactivity of XMMBR-B14 with CEA and NCA preparations was determined by a solid phase radioimmunoassay. Briefly, antigen preparations were coated into wells of microtest plates. Monoclonal antibody was then added, incubated 1 to 2 hours and wells washed. ^{125}I-labelled (F(ab)'2 fragments of rabbit anti-mouse IgG were then added to detect bound murine MoAb. Table VIII.

In the first test MoAb binding to semi-purified CEA (Rogers) and NCA (B3991) was compared. XMMBR-B14 bound to both NCA and CEA (NCA:CEA ratio 1.2:7).

TABLE VIII

Binding of XMMBR-B14 to CEA and NCA

Antibody	Mean CPM ± SD (- Background) Bound to:	
	CEA (Rogers)	NCA (B3991)
Anti-CEA	1427 ± 222	443 ± 48
XMMBR-B14	2558 ± 114	955 ± 140

E. XMMBR-B14 Dose Response on XMMCO-228-RTA Cytotoxicity Against MNK45 Cells

Table IX shows the increase in cytotoxicity of XMMCO-228-RTA immunotoxin against MNK45 cells when increasing amounts of XMMBR-B14 are added.

Six different curves were generated using concentrations of XMMCO-228-RTA ranging from 1 ng/ml to 100 ng/ml. To these concentrations were added varying amounts of XMMBR-B14 ranging from 0 to 10 µg/ml. The calculated IC_{50} values for each curve are shown in Table IX.

TABLE IX

XMMBR-B14 Produced Monoclonal Antibody
Dose Response on XMMCO-228-RTA Cytotoxicity
(MKN45 Cells)

XMMBR-B14 MoA (µg/ml)	Cytotoxicity of XMMCO-228-RTA for MKN45 Cells (IC_{50} ng/ml)
10	29
1	47
0.1	60.8
0.01	87
0.001	113
0.00	155

The present invention provides efficacious, novel compounds and methods for the therapy of various

cancer cells. The cytotoxic conjugates are particularly efficacious when administered in cocktail form or in the presence of certain unconjugated monoclonal antibodies.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for enhancing the cytotoxicity of an immunotoxin consisting of a first immunoglobulin conjugated to a toxin said first immunoglobulin directed against a cell surface antigen on a target cell - wherein the method comprises exposing a target cell expressing the antigen to the immunotoxin and a second immunoglobulin in a dose sufficient to potentiate said immunotoxin, said second immunoglobulin able to bind to a different epitope on the antigen than that bound by said first immunoglobulin.

2. A method according to claim 1, wherein the antigen is a tumor associated antigen.

3. A method according to claim 2, wherein the first and second immunoglobulins are able to bind to an epitope on a tumor cell expressing carcinoembryonic antigen.

4. A method according to claim 3, wherein the epitope bound by the second immunoglobulin is within the normal cross-reacting antigen subsection of the carcinoembryonic antigen.

5. A method according to claim 4, wherein the first immunoglobulin is XXMMCO-228 bound to RTA and the second immunoglobulin is XXMBR-B14.

6. The method of claim 1, wherein the first and second immunoglobulins are able to bind to an antigen on a T cell.

7. The immunoglobulins of claim 1, wherein the antigen is CD-5.

8. The immunoglobulins of claim 1, wherein the antigen is on a leukemic T cell.

9. A cytotoxic therapeutic cocktail comprising:

a conjugate comprising a toxin linked to a first immunoglobulin, said first immunoglobulin able to bind to an epitope on a cell surface antigen; and

a second immuloglobulin in a dose sufficient to potentiate said immunotoxin, said second immunoglobulin able to bind to a different epitope on the antigen than that bound by said first immunoglobulin.

10. A cocktail according to claim 9, wherein the antigen is a tumor associated antigen.

11. A cytotoxic therapeutic cocktail according to claim 9, wherein the first and second immunoglobulins are able to bind to an epitope on a tumor cell expressing carcinoembryonic antigen.

12. A cytotoxic therapeutic cocktail according to claim 11, wherein the epitope bound by the second immunoglobulin is within the normal cross-reacting antigen subsection of the carcinoembryonic antigen.

13. A cytotoxic therapeutic cocktail according to claim 12, wherein the first immunoglobulin is XXMMCO-228 bound to RTA and the second immunoglobulin is XXMBR-B14.

14. A cytotoxic therapeutic cocktail according to claim 9, wherein the first and second immunoglobulins are able to bind to an antigen on a T cell.

15. The immunoglobulins of claim 14, wherein the antigen is CD-5.

16. A method of killing cancer cells in humans by enhancing the cytotoxicity of an immunotoxin which consists of a first immunoglobulin conjugated to a toxin said first immunoglobulin directed against a tumor associated antigen - wherein the method comprises exposing cancer cells expressing the tumor associated antigen to the immunotoxin and to a second immunoglobulin in a dose sufficient to potentiate said toxin, said second immunoglobulin able to bind to a different epitope on the tumor associated antigen than that bound by said first immunoglobulin.

17. A method according to claim 16, wherein the first and second immunoglobulins are able to bind to an epitope on a tumor cell expressing carcinoembryonic antigen.

18. A method according to claim 17, wherein the epitope bound by the second immunoglobulin is within the normal cross-reacting antigen subsection of the carcinoembryonic antigen.

19. A method according to claim 18, wherein the first immunoglobulin is XXMMCO-228 bound to RTA and the second immunoglobulin is XXMBR-B14.

20. A method according to claim 16, wherein the cancer cells are selected from the group consisting of colorectal carcinoma cells, gastric cancer cells, pancreatic cancer cells, lung cancer cells, and breast cancer cells.

21. The method of claim 16, wherein the first and second immunoglobulins are able to bind to an antigen on a T cell.

22. The immunoglobulins of claim 21, wherein the antigen is CD-5.

23. The immunoglobulins of claim 21, wherein the antigen is on a leukemic T cell.

24. A method according to claim 16 wherein the ratio of said second immunoglobulin to said immunotoxin is 100 - 0.01 to 1 by weight.

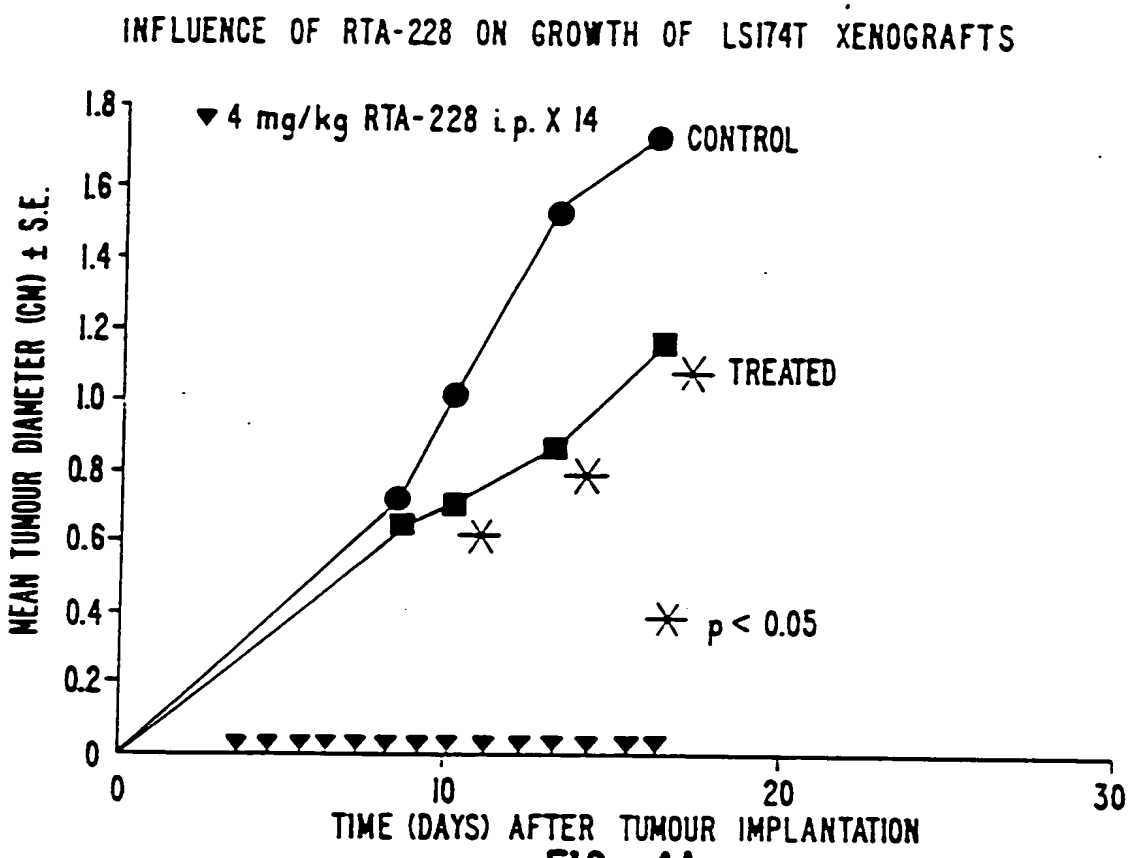
INFLUENCE OF RTA-228 ON GROWTH OF LS174T XENOGRAFTS
▼ 4 mg/kg RTA-228 i.p. X 14
CONTROL
TREATED
p < 0.05
MEAN TUMOUR DIAMETER (CM) ± S.E.
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
TIME (DAYS) AFTER TUMOUR IMPLANTATION

FIG_4A.

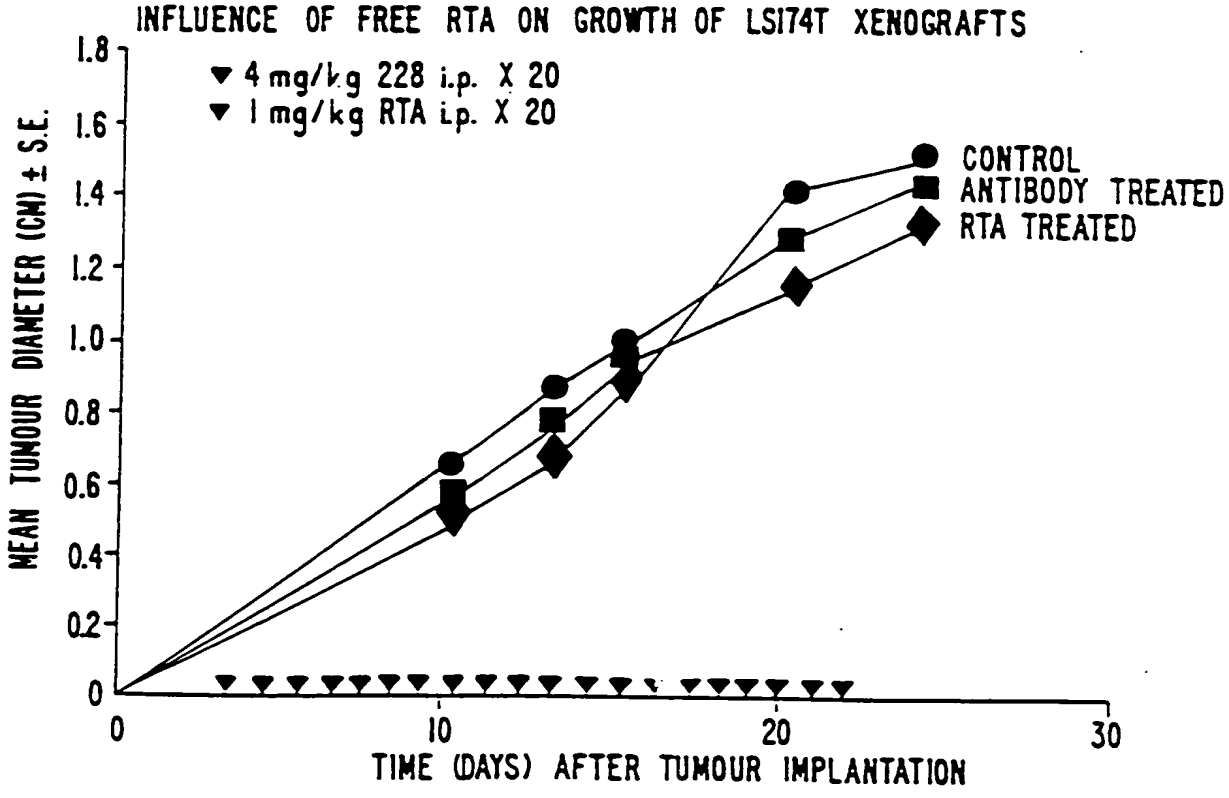
INFLUENCE OF FREE RTA ON GROWTH OF LS174T XENOGRAFTS
▼ 4 mg/kg 228 i.p. X 20
▼ 1 mg/kg RTA i.p. X 20
CONTROL
ANTIBODY TREATED
RTA TREATED
MEAN TUMOUR DIAMETER (CM) ± S.E.
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
TIME (DAYS) AFTER TUMOUR IMPLANTATION

FIG_4B.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/00243

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC 4: A61K 39/395
US CL: 424/85

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/85, 530/387, 391, 390, 388, 389

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

A.P.S., BIOSIS, CAS ON LINE (COMPUTER SEARCHES)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,590,071, (SCANNON ET AL) 20 MAY 1986, DEE ENTIRE DOCUMENT.	1-24
Y	EP, A, 0,098,162, (MATSUOKA ET AL), 30 JUNE 1984, SEE ENTIRE DOCUMENT.	1-24
Y	PROCEEDINGS OF THE NATIONAL ACADAMY OF SCIENCES, VOLUME 77, AUGUST 1980 (USA), "ANTIBODY-DIRECTED CYTOTOXIC AGENTS: USE OF MONOCLONAL ANTIBODY TO DIRECT THE ACTION OF TOXIN A CHAINS TO COLORECTAL CARCINOMA CELLS", SEE ENTIRE DOCUMENT.	1-24
Y	CHEMICAL ABSTRACTS, VOLUME 102, NO. 15, 15 APRIL 1985, (COLOMBUS, OHIO, USA), GRIFFIN ET AL, "ENHANCEMENT OF THE SPECIFIC CYTOTOXICITY OF ANTI-CARCINOEMBRYONIC CARBOXYLIC IONOPHORES," ABSTRACT NO. 130074n, PROTIDES BIOL. FLUIDS, (1984), 32, 449-53.	1-24

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 MARCH 1988	02 MAY 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	JEFF KUSHAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	CHEMICAL ABSTRACTS, VOLUME 104, NO. 19, 12 MAY 1986, (COLOMBUS, OHIO, USA), CASELLAS ET AL, "POTENTIATION OF CYTOTOXICITY INDUCED BY IMMUNOTOXINS", ABSTRACT NUMBER 161626q, UCLA SYMP. MOL. CELL. BIOL., NEW SER., 1985, 27, 263-74.	1-24